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Biotic and Abiotic Attenuation of Nitrogenous Energetic Compounds (NEC) in Coastal Waters and Sediments: Annual Report

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14. ABSTRACT

During the first year of this project, laboratory experiments were coupled with two field samplings; the first involved the temperate ecosystem of the Chesapeake Bay in March 2005, and the second involved the tropical ecosystem offshore of Oahu. In general, the study of the rates of abiotic transformation of NEC (Task 1) and photolytic degradation (Task 2) took place in the laboratory, while the study of the degradation by microalgae (Task 3) and heterotrophic bacteria (Task 4) took place in association with the field sampling. These results are described in the five peer-reviewed journal articles (Bhatt et al., 2005; Bhushanet et al., 2005; Cheng et al., 2006; Luning Prak and O'Sullivan, 2006; Monteil-Rivera et al., 2005; Monteil-Rivera et al., 2006), formal technical report (Walker et al., 2006), and invited abstract (Montgomery et al., 2005).

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Executive Summary

During the first year of this project, laboratory experiments were coupled with two field samplings; the first involved the temperate ecosystem of the Chesapeake Bay in March 2005, and the second involved the tropical ecosystem (Pacific Ocean) in May 2005. In general, the study of the rates of abiotic transformation of nitrogenous energetic compounds (NEC)(**Task 1**) and photolytic degradation (**Task 2**) took place in the laboratory, while the study of the degradation by microalgae (**Task 3**) and heterotrophic bacteria (**Task 4**) took place in association with the field sampling. These results are described in the six peer-reviewed journal articles (*Bhatt et al.* 2005, *Bhushan et al.* 2005, *Cheng et al.* 2006, *Luning Prak and O'Sullivan.* 2006, *Monteil-Rivera et al.* 2005, *Monteil-Rivera, et al.* 2006), formal technical report (*Walker et al.* 2006), and invited abstract (*Montgomery et al.* 2005).

The first field sampling took place primarily in the Chesapeake Bay during March 2005 and extended along the salinity gradient from the marine end member outside the mouth of the Chesapeake Bay to a freshwater station within the Potomac River. This sampling involved collecting seawater, sediment grabs and short cores for Tasks 1 and 4. The second field sampling took place offshore in the central Pacific Ocean in May 2005, and involved collecting seawater and sediment at two offshore stations where underwater demolition was being performed in separate detonations of 10 lbs of 2,4,6-trinitrotoluene (TNT) and 10 lbs of 1,3,5-trinitroperhydro-1,3,5-triazine (RDX). The demolition and sampling was performed with the support of the Explosives Ordnance Detachment Mobile Unit 3 (EOD MU03) at Naval Magazine Pearl Harbor (NAVMAG PH) in the tropical waters and surface sediment. We used a chase boat to sample the plume generated in the detonation over time, initially within 5 min of the detonation and then sampling every 15 min for an hour (as the plume dissipated and was no longer visible from the surface). *In situ* light attenuation profiles were collected, as well as, water samples for chemical analyses and biological experiments. We then used the surface water samples collected pre-detonation and immediately post-detonation in shoreside incubations in ambient light and temperature to determine the degradation rates of the residual TNT and RDX that were expected to result from the incomplete combustion of the detonation charges.

Our study of the abiotic transformation of NEC (**Task 1**) primarily involves laboratory experiments using both artificial media and field collected water and sediment samples with most of the work in the first year focusing on artificial media. The results in this task involved development of a method for trace analyses of NECs in marine environments, determining the rates of alkaline hydrolysis of NEC, and determining the NEC reduction rates by zero valent iron (ZVI). The analytical method that was developed involved coupling SPME/GC-ECD and optimizing the trace analyses for nine explosives, including nitroaromatics and RDX. Addition of a high concentration of sodium chloride (30 ‰) in the aqueous medium and use of a Carbowax/Divinylbenzene coating led to optimal extraction efficiencies. Method detection limits (MDLs) ranged from 0.05 to 0.81 $\mu g\ L^{-1}$ in water and from 1 to 9 $\mu g\ kg^{-1}$ in dry sediment.

The kinetics of RDX and 1,3,5,7-tetranitroperhydro-1,3,5,7-tetrazocine (HMX) hydrolyses were determined in deionized water and in a sodium chloride solution (30 ‰) at different pH's and temperatures. For both nitramines, hydrolysis was found to follow second-order kinetics with respect to the nitramine and OH^- concentrations. We obtained Ea = 113.6 kJ

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mol^{-1} and ln A = 44.1 L mol^{-1} min^{-1} for RDX, and Ea = 122.3 kJ mol^{-1} and ln A = 45.1 L mol^{-1} min^{-1} for HMX. From these values, half-lives ($t_{1/2}$) can be calculated at any pH and temperature (e.g., at 25°C and pH 8, $t_{1/2}$ (RDX) = 7 years and $t_{1/2}$ (HMX) = 99 years).

Finally, we investigated the potential of ZVI to transform HMX under anaerobic conditions. HMX underwent a rapid transformation when added in well-mixed anaerobic ZVI-H_2O batch systems to ultimately produce formaldehyde (HCHO), ammonium (NH_4^+), hydrazine (NH_2NH_2), and nitrous oxide (N_2O). Time course experiments showed that the mechanism for HMX transformation involved at least two initial reactions. One pathway involved the sequential reduction of N-NO_2 groups to the five nitroso products (1NO-HMX, cis-2NO-HMX, trans-2NO-HMX, 3NO-HMX, and 4NO-HMX). Another pathway implied ring cleavage from either HMX or 1NO-HMX as demonstrated by the observation of methylenedinitramine ($NH(NO_2)CH_2NH(NO_2)$) and another intermediate that was tentatively identified as $NH(NO_2)CH_2N(NO)CH_2NH(NO_2)$ or its structural isomer $NH(NO)CH_2N(NO_2)CH_2NH(NO_2)$. This was the first study to demonstrate transformation of HMX by ZVI to significant amounts of NH_2NH_2 and HCHO.

As a precursor to studying NEC photodegradation (**Task 2**) in seawater, we examined the effect of salt on the solubility of TNT and 2,4-dinitrotoluene (DNT). The presence of salt reduces the solubility of many organic compounds, in a process commonly called the "salting-out" or "salt" effect. The salting-out effect has been applied to transferring low-level concentrations of NEC from the aqueous phase to an organic phase for analysis, but very little research has been done on measuring the actual solubility of NECs in salt solutions. The solubility of 2,4-DNT and TNT in seawater (ionic strength = 0.3352, 0.5071, and 0.6820) and pure water was measured at 4°C, 20°C, 30°C, and 40°C. The pure water solubility values compare well with values reported previously and were higher than those for seawater. The average salting-out coefficients for 2,4-DNT and TNT were 0.11 and 0.12 L mol^{-1}, respectively, which are consistent with measurements for other nitroaromatic compounds. The salting-out coefficients did not vary significantly with temperature over the range examined.

Our study of biodegradation by microalgae (**Task 3**) in this first year was limited to processing seawater samples from the underwater TNT detonation in the Pacific Ocean field experiment. These seawater samples were incubated with ^{15}N-TNT under ambient light and collected for separation of the bacteria assemblage from phytoplankton by flow cytometry. Additional seawater samples were incubated with ^{14}C-TNT under ambient light conditions and collected for microautoradiographic fluorescent *in situ* hybridization (MicroFISH) of the microbial assemblage. Subsamples were taken at time 0, 24 h and 48 h and extracted for HPLC analyses of microalgal pigments for measurement of assemblage change with time after impact by the demolition charge. In addition, primary production was also measured in 24 and 48 h ambient light and temperature incubations. Although impact of the TNT detonation on rates of primary production appeared to be negligible, primary production was stimulated two-fold in the post RDX detonation samples after 24 h but returned to background levels within 48 h. These results suggest that the impact of underwater detonations on primary production may be transient.

Work on NEC biodegradation by bacteria (**Task 4**) involved surveys of the TNT, 2,4-

dinitrotoluene (DNT), and 2,4-diaminotoluene (DAT) mineralization in estuarine sediments, incorporation and mineralization rates in estuarine surface water, and laboratory culture work on 2,4-DNT, RDX and HMX transformation using environmental isolates. In the estuarine sediment surveys (which include our surveys from ONR and Y0817 programs for the past three years), TNT mineralization by the natural microbial assemblage was detected at 71% of the 56 stations and ranged up to 145 (+/- 16.0) μg C kg^{-1} d^{-1} though the median values of each survey ranged from 3.0 (+/- 0.56) to 17.6 (+/- 0.49) μg C kg^{-1} d^{-1}. The median values may be more useful for determining the capacity of ecosystem sediments to metabolize TNT (our **Task 5**). Despite the wide variation in ecosystem types (tropical versus temperate), the median TNT mineralization rates were within an order of magnitude among surveys. We also found that when averaging rates between stations where TNT mineralization was detected versus not detected, the average rates of bacterial production and mineralization of naphthalene, phenanthrene, fluoranthene, catechol and toluene were all higher at stations where TNT mineralization was detected. Also, the range in average PAH and toluene mineralization rates for all stations (0.80 to 8.04 μg C kg^{-1} d^{-1}) was very similar to the range of median TNT mineralization rates (3.08 to 17.6 μg C kg^{-1} d^{-1}). This suggests that TNT is mineralized in coastal sediments at rates that are typical for that seen with naturally occurring compounds like phenanthrene and fluoranthene. It is even similar to that seen with compounds like toluene and naphthalene that are known to be very transient in the marine and estuarine sediments.

In general, TNT mineralization rates by the natural bacterial assemblage in surface sediments were higher than those for 2,4-DNT and 2,6-DAT measured at the same station. The mineralization rate may relate to, but is not necessarily the same as, the amount of the parent compound that is removed from the environment. These higher mineralization rates could be due to a greater percentage of the active bacterial community metabolizing TNT, more efficient use of 2,4-DNT and 2,6-DAT by bacteria (higher % incorporation), or different cellular transport mechanism for TNT versus 2,4-DNT resulting in greater uptake rates for TNT. Estuarine surface water (Delaware Bay, March 2005) was incubated with ^{14}C-TNT, ^{14}C-DNT and ^{14}C-DAT to measure the incorporation and mineralization rates and growth efficiencies of free-living bacteria metabolizing these compounds. Bacteria among the natural assemblage had higher rates of both incorporation and mineralization for TNT relative to 2,4-DNT and 2,6-DAT. Bacteria incorporated 2,4-DNT at a lower rate and with lower efficiency than either TNT or 2,6-DAT. The growth efficiencies of 98% for 2,6-DAT and 90% for TNT are extremely high for aromatic carbon sources in marine environments. A new hypothesis derived from these results is that nitroaromatics like TNT and 2,6-DAT are very efficiently incorporated into bacteria macromolecules (e.g. proteins or DNA). The mineralization rates that we measure for the sedimentary assemblages may actually be the result of remineralization of bacterial macromolecules by protozoan grazers.

In addition to the field work on natural assemblages, laboratory experiments were performed using bacterial monocultures isolated from impacted marine environments. One marine bacterium, *Clostridium* sp. EDB2, was isolated from off of Halifax, Nova Scotia and could degrade cyclic nitramines in the presence of Fe(III), humic acids (HA), and anthraquinone-2,6-disulphonate (AQDS). This obligate anaerobe degraded RDX and HMX to nitrite, methylenedinitramine, nitrous oxide, ammonium, formaldehyde, formic acid, and carbon dioxide. Carbon and nitrogen mass-balance for RDX products were 87 and 82 %, respectively,

and for HMX were 88 and 74 %, respectively, suggesting that incorporation efficiencies ranged from 12 to 26%. Bacterial growth and biodegradation of RDX and HMX were stimulated in the presence of Fe(III), HA, and AQDS suggesting that strain EDB2 utilized Fe(III), HA, and AQDS as redox-mediators to transfer electrons to cyclic nitramines. This strain demonstrated a multidimensional approach to degrade RDX and HMX: first, direct degradation of the parent compound; second, indirect degradation by reducing Fe(III) to produce reactive-Fe(II); third, indirect degradation by reducing HA and AQDS which act as electron-shuttles to transfer electrons to the cyclic-nitramines.

Laboratory work was also performed to understand the bacterial biodegradation of 2,4-DNT by a novel marine bacterium, *Pseudoxanthomonas* sp. JA40, isolated from the Johnston Atoll in the Pacific Ocean. JA40 is hypothesized to degrade 2,4-DNT because it evolved in an environment deficient of organic nitrogen sources but rich in polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). This facultative anaerobe was able to transform 2,4-DNT both aerobically (with the production of metabolites) and anaerobically, using this NEC as both a carbon and nitrogen source. RDX and HMX were also degraded using anaerobic enrichment cultures from the marine sediment samples from the Oahu UXO field. These mixed cultures mineralized 69% or 57% (25 d) of the total carbon of ^{14}C-RDX or ^{14}C-HMX, respectively. The most abundant members of the consortium (based on PCR-DGGE) were γ-proteobacteria (*Halomonas*), sulfate-reducing δ-proteobacteria (*Desulfovibrio*), firmicutes (*Clostridium*), and fusobacterium. Among 22 sediment bacterial isolates screened for RDX and HMX biodegradation activity under anaerobic conditions, 5 were positive for RDX and identified as *Halomonas*, *Marinobacter*, *Pseudoalteromonas* and *Bacillus* based on 16S rRNA. Sediment bacteria degraded RDX to N_2O and HCHO via the intermediate formation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and methylenedinitramine.

Thus far, it appears as though abiotic hydrolysis of NEC in seawater occurs at a rate that is orders of magnitude slower (years) than transformation rates by culturable, anaerobic bacteria and than mineralization rates by natural microbial assemblages (days). The second year of this project will focus more on the mechanisms of microbial degradation of NEC, as well as, the comparison to the rates of photodegradation. Preliminarily, it appears that natural attenuation processes have the potential to remove NEC released into the marine environment on a time scale that may be useful in standard risk assessment analyses of impacted sites.

Objective

The objective of this research proposal is to characterize the fate of NEC (TNT, RDX, and HMX) released from munitions in submerged coastal waters and sediments. Specific issues that will be addressed include assessment of the potential for underwater degradation and transport of such residues in fresh, brackish and salt water environments that are representative of Department of Defense (DoD) water training and testing ranges. The specific goals of this proposal are to:

1. Determine the rates of dissolution, phase transformation, abiotic degradation (hydrolysis, reduction), photodegradation and microbial transformation under various environmental conditions (e.g. salinity, light intensity, nutrient availability and limitation, total organic carbon (TOC) concentration and type, iron concentration) that are likely to be important at DoD sites.

2. Predict time scale of attenuation of NEC and potential for transport offsite to adjacent ecosystems by modeling transformation rates under expected environmental conditions for three types of UXO-impacted areas: subsurface detonation area, operational range, historic dumpsites.

This work will consist of laboratory and mesocosm experimentation, as well as field surveys and experimentation to provide the rate measurements and assessment of typical environmental conditions found at DoD coastal sites impacted by NEC. The rates for photochemical and biological degradation of NEC in the water column, and biological degradation in the sediments, will support predictive models of overall NEC degradation in the coastal environment which may be used by site managers for in-place NEC remediation strategies. The utility of these models is that they shall be derived empirically and verified by our laboratory and mesocosm experiments and field surveys.

Background

Currently, the Navy has approximately 230 acres of active and inactive ranges that are underwater and facing response action due to presence of UXO (RITS 2001 seminar series). However, the Navy has about 1.29 million acres of active/inactive ranges in their entire inventory. The future cost to DoD of environmental compliance to maintain these ranges may preclude their future use. This inventory includes coastal sites in Hawaii, California, Alaska, Japan, Louisiana, and Okinawa. Although SERDP, Army and Air Force have sponsored much work on energetic transformation in terrestrial and groundwater systems, very little information is available on the attenuation or transport of energetics in coastal aquatic systems. In lieu of evidence that these materials are rapidly degraded to nontoxic substances or metabolized by microbial assemblages, DoD is being pressured to recover all UXO from coastal waters. Recently, we determined that biodegradation and photodegradation rates of TNT in marine waters are surprisingly rapid compared to the rates in terrestrial systems. This information may help DoD maintain access to critical operational ranges and reduce unnecessary clean up costs.

Materials and Methods

i) **Dissolution, Phase Transformation, and Abiotic Degradation** To evaluate the dispersion of energetic compounds upon subsurface detonation, both water column (particulate and dissolved) and sediment (surface) samples will be analyzed by NRL-DC. To fully characterize the dispersal of the compounds, upper, mid, and lower water column depths and surface sediment will be sampled immediately post-detonation (within 1 h), and every 8 h for the next 24 hours. Subsequent sampling will be conducted once a day for the next five days. A 1 L volume of seawater will be collected from upper, mid, and lower water column depths within the area of detonation via Niskin bottle collection. The water column samples will be filtered for particulates within 10 h of collection. Surface sediment samples will be collected via surface grab sampler. Dissolved phase seawater samples will be kept cold with minimal headspace during transport and extracted immediately upon return to the laboratory. Sediment and filtered particles will be frozen until analysis.

In recently used ranges and historic dumpsites, limited water column samples will be collected following the protocol below. Additionally, benthic nepheloid samples will be obtained and processed following the water column sample protocol (collection of both dissolved and particulate phases). Sediment cores will be collected using a box corer to assess both surface and sub-surface environments in a grid pattern within the area of disposal/detonation. Cores will be extruded and sub-sampled at 2 cm intervals within 48 h of core collection. Sediments will be centrifuged to separate porewater from each sub-section immediately after collection with porewaters filtered to remove any particulates. Dissolved and particulate samples for all study sites will be extracted per USEPA Method 8330 for nitroaromatics and nitroamines and analyzed by HPLC. In addition to energetics analysis, other parameters will be evaluated using standard protocols including: dissolved and particulate organic carbon (water column), total suspended solids (water column), chlorophyll (water column and sediment), total organic carbon and nitrogen (sediment), grain size (distribution) and specific surface area (sediment).

Hydrolysis kinetics will be measured in synthetic solutions for TNT, RDX and HMX at different temperatures, pH and ionic strengths. They will then be measured at different temperatures in water column samples collected from the study sites. Kinetics data (rate constants, Arrhenius factors and activation energies) will then be deduced from the laboratory data to predict the hydrolysis rates and half-life times ($t_{1/2}$) of each explosive at the sampled sites. RDX and HMX reaction with zerovalent metals (*e.g.* Fe(0)) will be conducted under various conditions (pH, Eh, O_2). Degradation rates and stoichiometry will be determined to interpret degradation in sediments. The presence of RDX and HMX hydrolysis products (NO_2^-, $HCOO^-$, and NDAB) in the pore water of contaminated sediments or in the water column of detonation area will be verified. To assess the abiotic degradation of RDX and HMX by ferrous iron, we will use iron (III) oxide minerals (e.g., hematite, goethite,) in the presence of iron-reducing bacteria isolated from sediments. Reaction kinetics and stoichiometry will be determined for RDX and HMX degradation and used to estimate the contribution of reduction by ferrous minerals in sediments. To verify the interaction of humics with RDX and HMX, commercially available humic acid and other electron shuttles such as anthraquinone-2,6-disulphonate (AQDS) will be used as electron acceptors for the humic acids-reducing bacteria. The reduced substrates will be tested for RDX and HMX degradation and results will be used to interpret degradation in

sediment. In all the above experiments, NEC will be analyzed by reverse phase HPLC with a CN column and a photodiode array detector (λ 230 nm). The potential end-products, ammonium cation (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and formate ($HCOO^-$) anions will be analyzed by ionic chromatography. N_2O and N_2 will be analyzed from the head-space by GC with an electron capture detector (ECD) and a thermal conductivity detector (TCD), respectively. Formaldehyde (HCHO) will be analyzed by RP HPLC (LC-8 column; fluorescence detector) after derivatization with 2,4-pentanedione. NDAB and methylenedinitramine will be analyzed by HPLC. Ring labeled ^{15}N-RDX and ^{15}N-HMX will be used to determine C and N mass balances and reaction stoichiometries for degradation pathways.

ii) **Photodegradation** USNA will measure Fe and peroxides in samples collected from field surveys and during experimental manipulations. O'Sullivan's lab at USNA has developed techniques to determine the speciation of iron in natural waters at nanomolar levels (*O'Sullivan et al.* 1991, 1995, 1997), and has extensive experience in peroxide analyses in natural systems (*O'Sullivan et al.* 1999, 2004). NRL-DC will measure CDOM absorption in water samples by UV-vis spectrophotometry and will characterize CDOM by synchronous fluorescence. Production of nitrite by photochemical degradation of TNT will be measured by UV-vis spectroscopy on reaction with sulphanilamide in acidic solution followed by reaction with N-1-napthyl-ethylenediamine to form an azo dye. Nitrate will be measured in the same way after reduction on a Cd column. NRL-DC will extract humics with C18 resin and measure extracted humics as organic carbon by persulfate oxidation. TOC on aqueous phases will also be measured by persulfate oxidation. NRL-DC will also measure light attenuation in the water column and surface irradiance with a Biospherical Instruments PUV-501b profiling UV radiometer (*Osburn et al.* 2001, *Morris and Hargreaves* 1997). NRL-DC and USNA will use sunlight incubations of model compounds in filtered surface water samples to determine the kinetics of the photochemical degradation of TNT under ambient conditions and under various concentrations of humics, CDOM, and Fe. In addition to kinetic studies, NRL-DC and USNA will also calculate the quantum yield for photochemical degradation of TNT, HMX, and RDX in seawater under ambient conditions and with variations in humics, CDOM, and Fe. Quantum yields will be calculated following published methods for photobleaching (*Osburn et al.* 2001); thus they will be based on measured light (milliWatts cm^{-2} nm^{-1}). The degradation rate will be measured by radiotracer assays of substrate mineralization using UL-^{14}C-TNT, -DNT, and -RDX added to quartz tubes and exposed either to sunlight or in the solar simulator (*Boyd et al.* 1996).

iii) **Transformation by Phytoplankton** Total microplankton abundance will be determined via direct microscopic counts (*Hobbie et al.* 1977). Phytoplankton and cyanobacteria will be differentiated from bacteria using bright field microscopy and autofluorescence. Bioquant ™ Image Analysis will be used for abundance counts, and to identify and enumerate indicator organisms (e.g., *Anabaena* sp.)(*Hamdan* 2003). Phytoplankton community structure will be characterized using pigment analysis by UNC according to *Paerl et al.* (2003). Algal pigments will be separated and identified using HPLC and in-line photodiode array spectrophotometry detection. Phytoplankton functional groups (PFG) will be determined based on separation and quantification of photopigments. If algal and bacterial abundances and incorporation rates are high enough, we will attempt to identify individual species or groups of microorganisms with affinity for ^{15}N-labeled NECs. This will be accomplished by NRL-DC using flow cytometry (*Winson and Davey* 2000). Cells will be sorted based on fluorescence (in

as many discriminatory fractions as possible) analyzed taxonomically, then concentrated and analyzed by IRMS.

To assess the use of NECs as a source of N to the biota (particularly in oligotrophic environments), a series of water column samples will be incubated using tracer additions of ^{15}N-labeled NECs. Incubations will be conducted under dark, ambient light and surface light conditions with natural microplanktonic organisms to assess the combined role of solar radiation and microbial processes on NEC utilization. Cell specific incorporation of NECs (TNT, RDX and HMX) will be surveyed by UNC using microautoradiography (*Paerl* 1984) to determine importance of heterotrophs vs. autotrophs in NEC degradation. Incorporation of ^{15}N-labeled NEC will be tracked into bacterial and algal fractions to determine the fate of N from NECs (*Neess et al.* 1962). After incubating under *in situ* conditions, algal and bacterial cells will be collected using differential filtration. The collected cells will be converted to N_2 and introduced into an Isotope Ratio Mass Spectrometer (IRMS) to quantify the amount of ^{15}N associated with algal and bacterial populations. Incorporation and production rates will also be tracked in treatment groups amended with alternate N (inorganic and organic), phosphorous (P) or N+P sources to resolve if NEC is inhibited. This will provide an understanding of the importance of available NEC to the microplankton community. Whole community primary production will be measured as the uptake of $NaH^{14}CO_3$ into particulate and dissolved fractions (*Parsons et al.* 1984). Production (primary and secondary) and metabolism (*Smith and Azam* 1992, *Jonas et al.* 1988) will be measured in samples exposed to varying concentrations of NEC (unlabeled) to determine stressful effects on microplankton from different locations.

iv) **Transformation by Heterotrophic Bacteria** NRL-DC will perform NEC mineralization assays which will be initiated within three hours of water or sediment sample collection using a modification of *Boyd et al.* (1996) and *Pohlman et al.* (2002). These methods involve radiotracer additions of ^{14}C-TNT, -RDX and -HMX to surface sediment samples or water samples and incubated (24 h), evolved $^{14}CO_2$ will be captured, radioassayed and subsequently used to calculate substrate mineralization. The leucine incorporation method (*Kirchman et al.* 1985, *Kirchman* 1993, *Smith and Azam* 1992) will be used to measure bacterial production as adapted by *Montgomery et al.* (1999). Leucine incorporation rate will be converted to bacterial carbon using factors determined by *Simon and Azam* (1989).

Bacterial communities in both the water column and sediment samples will be characterized by integrated cultivation and molecular DNA techniques. These approaches will analyze genetic diversity of complex populations. Methods to be used will include DNA polymerase chain reaction (PCR), cloning, denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH), and numeration by colony counting and the most-probable-number method (*Rooney-Varga et al.* 1999; *Daims et al.* 2001; *Nogales et al.* 2001; *Watts et al.* 2001; *Bouvier and del Giorgio* 2003; *Ivanov et al.* 2003; *Sekar et al.* 2003; *Zwirglmaier et al.* 2004). Bacterial community DNA will be amplified by PCR using specific primers. A 16S rRNA bacterial specific forward primer (27F) and a universal reverse (1492R) will be used for cloning, and bacterial specific 16S rRNA primers (b341GC, and b758) for DGGE (*Delong* 1992; *Juck et al.* 2000). Archaeal rRNA will be similarly amplified with Archaeal specific primers (21F and 958R) (*Delong* 1992). The DGGE primer set amplifies a region ca. 500 bp that corresponds to positions 341-758 in *Escherichia coli* and resulting PCR

products will be analyzed with the DCode system (Bio-Rad). Resulting clones and well-separated bands from the DGGE will be sequenced in our lab with an Amersham MegaBace 1000 DNA sequencer. Sequences are used for identification and subsequent phylogenetic determination by comparative sequence analysis. DGGE banding patterns among samples are compared to determine community structural differences, which can indicate relative abundances of the main bacterial populations within different samples.

The rates of bacterial production and NEC mineralization will be used to determine a mass balance for heterotrophically metabolized NEC added to a system either directly added to a test tube, or mesocosm enclosed system, or estimated release from the UXO into the sediment and overlying water column in the case of a field site. The production and mineralization rates will be correlated with ambient concentrations of TOC, nitrogen and phosphorus species, and depth of bioturbation (mesocosms only). The rates of attenuation by heterotrophic bacteria will be compared with those by phytoplankton and photolysis. The competition for NEC-derived N with cyanobacteria will also be compared to determine the relative importance of each at the field sites and within the mesocosms.

v) **Mesocosm Experiments** NRL-Stennis will support laboratory mesocosm studies to determine NEC fate within a system that has more complexity than benchtop laboratory experiments but is more controlled than the subsequent field studies proposed for DoD sites. The remineralization rate of energetic compounds within the geochemical regimes commonly found in aquatic sediments will be quantified and related to the 3D distribution of redox species and their spatial and temporal oscillations using a series of benthic mesocosm experiments (*Furukawa et al.* 2001). In one set of experiments, NEC is introduced to the water-sediment interface in the mesocosm whose redox regimes has been established and reached steady state. NEC will be transported by bioturbation and diffusion, as well as remineralization. The reaction and transport will be monitored by the time-series sampling and analysis of pore water (organic acid, nitrogen compounds, phosphorus compounds, O_2 and SO_4^{2-} concentrations) and sediment particles (bacterial cell density, Fe(II)/Fe(III) ratio). In the other set of experiments, NEC is incorporated into the sedimentary substrates by mixing at the time of mesocosm loading. The gradual establishment of redox regimes and concurrent transport and mineralization of NEC will be monitored. The biogeochemical reaction-transport model with explicit treatment of biologically-induced redox oscillation (*Furukawa* 2001) will be used to further quantify the redox regimes in mesocosms, as well as in the field sites. The concurrent mesocosm experiments and numerical modeling of the reaction and transport within the system will give us a tool to interpret the batch incubation experiments (explained above) in realistic contexts.

vi) **Field Sites** Three types of field sites are proposed to provide a variety of environmental situations with regards to length of exposure to NEC; underwater detonation sites, UXO dumpsites, and active/inactive ranges. These sites may or may not be under the regulatory oversight of compliance rather than cleanup, but they are chosen based on their accessibility, usefulness in providing a scientific platform of study, availability of site support for cost wedging, and likelihood that site specific information provided in this study will be directly useful to the DoD installation.

vii) **Water Column Modeling** This area involves incorporating field site and mesocosm parameters and NEC mineralization rates into a 2D model in coordination with another SERDP project. Photic zone depth and wavelength information will be collected during DoD field site surveys and compared with NEC mineralization rates by bacteria and phytoplankton. Other site parameters will be collected as part of the surveys including those whose effects are measured in task areas 1-3 (e.g. salinity, DOC, POC, temperature, iron concentration. Models will be made for water column and surface sediment for three types of sites, underwater detonation, recently used range and historical range. A model for NEC degradation with depth in sediment will also be developed based on current versions involving changing electron acceptor concentration in the mesocosm experiments**.**

Results and Accomplishments

During the first year of this project, laboratory experiments were coupled with two field samplings; the first involved the temperate ecosystem of the Chesapeake Bay in March 2005, and the second involved the tropical ecosystem offshore in the central Pacific Ocean in May 2005. In general, the study of the rates of abiotic transformation of NEC (**Task 1**) and photolytic degradation (**Task 2**) took place in the laboratory, while the study of the degradation by microalgae (**Task 3**) and heterotrophic bacteria (**Task 4**) took place in association with the field sampling. The results from the first year are largely described in the five peer-reviewed journal articles, formal technical report, and invited abstract listed below:

A) *Bhatt M., Zhao, J.-S., Monteil-Rivera, F., and J. Hawari.* 2005. Biodegradation of cyclic nitramines by tropical marine sediment bacteria. J. Ind. Microbiol. Biotechnol. 32: 261-267.

B) *Bhushan, B., Halasz, A., and J. Hawari.* 2005. Effect of iron(III), humic acids, and anthraquinone-2,6-disulphonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. J. Appl. Microbiology (*in press*).

C) *Cheng, J. Z., Harada, R., Campbell, S., and Q. X. Li.* 2006. Biodegradation of dinitrotoluene by *Pseudoxanthomonas* sp. JA40. J. Young Investigators. (*in submission*)

D) *Luning Prak, D. J., and D. W. O'Sullivan.* 2006. Solubility of 2,4-Dinitrotoluene and 2,4,6-Trinitrotoluene in seawater. J. Chem. Ref. Data (*in press*)

E) *Monteil-Rivera F., Beaulieu C., and J. Hawari.* 2005. Use of solid phase microextraction/gas chromatography-electron capture detection for the determination of energetic chemicals in marine samples. J. Chromatogr. A 1066: 177-187.

F) *Monteil-Rivera, F., Paquet, L., Halasz, A., Montgomery, M. T., and J. Hawari.* 2006. Reduction of Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by Zerovalent Iron: product distribution. Environ. Sci Technol. 39:9725-9731.

G) *Montgomery, M. T., Osburn, C. L., Boyd, T. J., Hamdan, L. J., and S. E. Walker.* 2005. Biodegradation of nitrogenous energetic compounds in coastal ecosystems. Presentation at the 26th Annual Meeting of SETAC North America, Baltimore, MD, November 13-16. (INVITED)

H) *Walker, S. W., Osburn, C. L., Boyd, T. J., Hamdan, L. J., Coffin, R. B., Smith, J. P., Li, Q. X., Hennessee, C., Monteil-Rivera, F., Hawari, J., and M. T. Montgomery.* 2006. Mineralization of 2,4,6-trinitrotoluene in coastal waters and sediments. US Naval Research Laboratory Formal Report, NRL/FR/6114—06-XXXX. (*in preparation*)

The first field sampling took place along a Chesapeake Bay transect during March 2005 which extended along the salinity gradient from the marine end member outside the mouth of the Chesapeake Bay to freshwater within the Potomac River (Figure 1). Estuarine water was also

collected at the mouth of the Delaware Bay for some shipboard experiments (aboard the R/V Cape Henlopen, Lewes, DE) on TNT uptake, incorporation and mineralization rates by natural bacterial assemblages. Estuarine sediment and water was collected and shipped for use in the abiotic NEC transformation laboratory experiments, as well as for the mesocosm experiments. Surface sediment was collected at 15 stations along the salinity gradient for shipboard incubations to measure mineralization rates of TNT, 2,4-DNT and 2,6-DAT by natural bacterial assemblages.

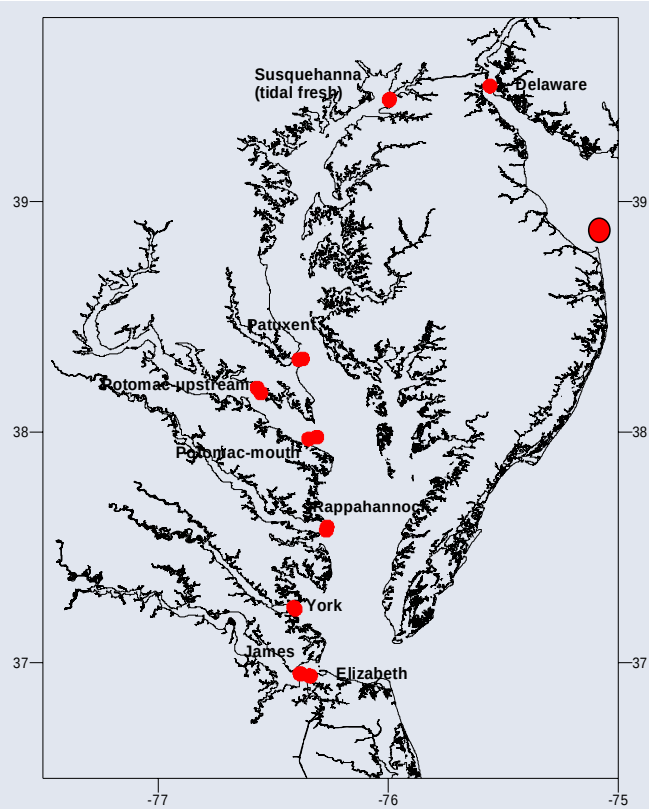

Figure 1. Chesapeake Bay station locations for March 2005 sampling aboard the R/V Cape Henlopen.

In May 2005, we sampled seawater and sediment at two offshore stations where underwater demolition was taking place in separate detonations of 10 lbs of TNT and 10 lbs of RDX (Figure 2). The demolition and sampling was performed with the support of the EOD MU03 at NAVMAG PH in the tropical waters and surface sediment offshore in the central Pacific. We also used a chase boat to sample the plume generated in the detonation over time, initially within 5 min of the detonation and then sampling every 15 min for an hour (as the plume dissipated and was no longer visible from the surface). *In situ* light attenuation profiles were collected, in addition to seawater samples for chemical analyses and biological experiments. We then used the surface water samples collected pre-detonation and immediately post-detonation in shoreside incubations in ambient light and at *in situ* temperature (27^{o}C) to determine the degradation rates of the residual TNT and RDX that were expected to result from the incomplete combustion of the detonation charges.

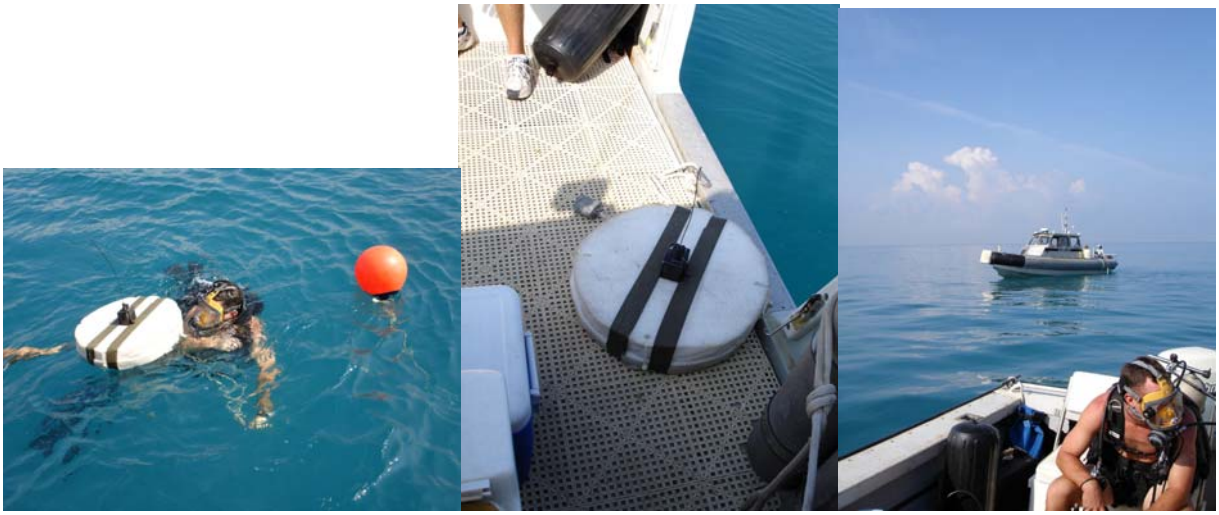

Figure 2. (L to R) Divers deploying the radio receiver to detonate the demolition charge; radio receiver on float; chase boat (background) for sampling the plume and dive boat (foreground).

Task 1. Abiotic Transformation

The abiotic transformation of NEC task primarily involves laboratory experiments on both artificial media and field-collected water and sediment samples with most of the work in the first year focusing on artificial media.

Subtask 1.1 Determine distributions of NECs in field samples

The objectives of this subtask were met through development of a novel analytical method for measurement of low concentration of NECs in the marine environment and through the attempted measurement of NECs in the seawater of the plume from the underwater detonation of 10 lbs of RDX and 10 lbs of TNT as part of a demolition exercise. Although this task is listed as completed, further work will be performed on additional field sites as they become available.

Method Development Though water column samples can be analyzed using US EPA SW-846 method 3535A (solid phase extraction (SPE)) with either HPLC or GC detection, analysis of sediment and pore water required special attention due to the presence of organic interferences in sediments and the limitation of volumes of pore water. An analytical method was developed to determine distribution of NECs at trace levels in pore water and sediment samples. Gas chromatography with electron capture (GC-ECD) is a highly explosive-sensitive detection technique. However, its application to the analysis of sediment extracts is often hampered by numerous endogenous interferences so solid phase microextraction (SPME) was used to purify sediment extracts. The use of SPME, which involves a miniature cylindrical coated fused-silica fiber capable of adsorbing analytes from a smaller volume of solution (relative to SPE) was particularly attractive for the analysis of pore water samples.

SPME/GC-ECD coupling was optimized and applied to the trace analysis of nine explosives including nitroaromatics and RDX in field-collected seawater and marine sediment

samples. Addition of a high concentration of sodium chloride (30 ‰) in the aqueous medium and use of a Carbowax/Divinylbenzene coating led to optimal extraction efficiencies. Method detection limits (MDLs) ranged from 0.05 to 0.81 μg L^{-1} in water and from 1 to 9 μg kg^{-1} in dry sediment. Except for RDX, spike recoveries in seawater were satisfactory (89-147 %) when samples were fortified at 2 μg L^{-1} of each analyte. Spike recoveries from dry sediment (10 μg kg^{-1} analyte additions) had lower recoveries but these could also be due to degradation into the matrix. In addition to reducing the necessary sample volume, the use of SPME eliminated interferences present in sediment extracts thus allowing the detection of the target analytes that were otherwise difficult to detect by direct injection. This method will be used to analyze sediment samples from future sites in the Chesapeake Bay, Hawaii, and Puget Sound. This work is more completely described in the peer-reviewed publication (*Monteil-Rivera et al.* 2005).

Field Measurements The distribution of TNT, RDX and their degradation products was measured using LC-MS on seawater samples from the plumes of two underwater demolitions in the Pacific Ocean. The detonations were temporally and spatially separate; the first involved 10 lbs of TNT while the second consisted of 10 lbs of RDX. Surface water (1 m below air-sea interface) and sediment (top 6 cm) were collected prior to the detonation (ca. 20 min) and immediately afterwards (5 min and then every 15 min for surface water until the plume was no longer visible from the surface). Surprisingly, NEC concentrations were below the detection limit (ca. 0.6 μg L^{-1}) using LC-MS in all water samples for both detonations. Sampling was performed in the obvious debris field in the water column but no NEC compounds were detected. The efficiency of the underwater explosions greatly exceeded our expectations. This field experiment portion of the subtask is described in *Walker et al.* (2006).

Subtask 1.2 Determine extent and rates of alkaline hydrolysis

The kinetics of RDX and HMX hydrolyses were determined in deionized water at different pH's and temperatures. For both nitramines, hydrolysis was found to follow second-order kinetics with respect to the nitramine and OH^- concentrations. Second-order kinetics could be assimilated to pseudo-first order kinetics by using excess OH^-. Second-order rate constants (k2) were therefore calculated from pseudo-first-order rate constants (k1) using the following equations:

$$\ln (C_t/C_0)) = -k1\ t, \text{ and } k2 = k1/COH^- \qquad (1)$$

where:
C_0 and C_t are the concentration of the target compound at initial time and instant t, respectively,
k1 is the pseudo-first order rate constant at a given pH, and
k2 is the second order rate constant, and COH^- is the excess concentration of hydroxide employed.

Measuring k2 at different temperatures (T) allowed determining the Arrhenius factor A and the activation energy Ea that are related by the equation: ln k2 (T) = ln A – Ea/RT. We obtained Ea = 143.6 kJ mol^{-1} and ln A = 55.4 L mol^{-1} min^{-1} for RDX, and Ea = 132.1 kJ mol^{-1} and ln A = 48.4 L mol^{-1} min^{-1} for HMX. From these values, half-lives ($t_{1/2}$) can be calculated at

any pH and temperature. For instance, at 25°C and pH 8, $t_{1/2}$ (RDX) = 17 years and $t_{1/2}$ (HMX) = 130 years.

Additional hydrolysis experiments were conducted in the presence of NaCl (30 ‰) to mimic natural conditions in seawater. Sodium chloride did not affect the kinetics of degradation for both chemicals. Presently, kinetics experiments are being conducted using sterilized estuarine from Chesapeake Bay. Kinetics of hydrolysis of TNT was also measured in deionized water under various conditions of pH and temperature. Several initial products were identified including a UV absorbing complex that may be the Meisenheimer Complex, trinitrobenzene. Some efforts are being made to model the kinetics of TNT degradation in deionized water.

Subtask 1.3 Reduction of TNT, RDX, HMX by Fe(0) and Fe(II)

In contrast to RDX, HMX tends to accumulate in soils. In this subtask, we investigated the potential of ZVI to transform HMX under anaerobic conditions. HMX underwent a rapid transformation when added in well-mixed anaerobic ZVI-H_2O batch systems to ultimately produce formaldehyde (HCHO), ammonium (NH_4^+), hydrazine (NH_2NH_2), and nitrous oxide (N_2O). Time course experiments showed that mechanism for HMX transformation occurred through at least two initial reactions. One pathway involved the sequential reduction of N-NO_2 groups to the five nitroso products (1NO-HMX, cis-2NO-HMX, trans-2NO-HMX, 3NO-HMX, and 4NO-HMX). Another pathway implied ring cleavage from either HMX or 1NO-HMX as demonstrated by the observation of methylenedinitramine ($NH(NO_2)CH_2NH(NO_2)$) and another intermediate that was tentatively identified as ($NH(NO_2)CH_2N(NO)CH_2NH(NO_2)$) or its isomer. This is the first study that demonstrates transformation of HMX by ZVI to significant amounts of NH_2NH_2 and HCHO. Both toxic products seemed to persist under reductive conditions, thereby suggesting that the ultimate fate of these chemicals, particularly hydrazine, should be understood prior to using ZVI iron to remediate cyclic nitramines. This work was accepted for publication in a peer-reviewed journal (*Monteil-Rivera et al.* 2005).

Task 2. Photodegradation

Subtask 2.1 Determine effect of salinity

The presence of salt has been found to reduce the solubility of many organic compounds, and this process is commonly called the “salting-out” or “salt” effect. The salting-out effect has been applied to transferring low-level concentrations of nitroaromatic compounds from the aqueous phase to an organic phase for analysis, but very little research has been done on measuring the actual solubility of nitroaromatic compounds in salt solutions. For the organic compounds whose solubility in salt solutions have been measured, the Setschenow equation is commonly used to correlate organic compound solubility measurements to salt concentration (C_{salt} in moles L^{-1}):

$$\log (S_w/S) = K_s C_{salt} \qquad (2)$$

where S_w is the solute solubility in pure water (moles L^{-1}), S is the solute solubility in the salt solution (moles L^{-1}), and K_s is the salting-out parameter (L mol^{-1}), which is often referred to as

the Setschenow coefficient. A modified version of this equation correlates organic compound solubility to ionic strength (I in moles L^{-1}):

$$\log (S_w/S) = K_s' I \quad (3)$$

where K_s' is a modified salting-out parameter (L mol^{-1}). The latter equation was used in this study because seawater is a complex mixture of salts. *Hashimoto et al.* (1984) reported K_s' values ranging from 0.136 L mol^{-1} to 0.163 L mol^{-1} for monosubstituted nitrophenols and nitrotoluene in sodium chloride solutions. The goal of this research was to measure the solubility of 2,4-DNT and TNT in seawater over a range of salinity encompassing most coastal waters and at various temperatures.

The solubility of 2,4-DNT and TNT in seawater (ionic strength = 0.3352, 0.5071, and 0.6820) and pure water was measured at 4°C, 20°C, 30°C, and 40°C. The pure water solubility values compare well with values reported previously and were higher than those for seawater. The average salting-out coefficients for 2,4-DNT and TNT were 0.11 and 0.12 L mol^{-1}, respectively, which are consistent with measurements for other nitroaromatic compounds. The salting-out coefficients did not show any significant variation with temperature over the range examined. Details of these experiments have been submitted for publication (*Luning Prak and O'Sullivan* 2006).

Task 3. Microalgal Degradation

Subtask 3.1 Microautoradiography with ^{14}C-NEC and cyanobacteria

Water samples collected from the plume of the underwater TNT detonation were incubated with ^{14}C-TNT under ambient laboratory light conditions and collected for subsequent microautoradiographic fluorescent *in situ* hybridization (MicroFISH) of the microbial assemblage. Subsamples were taken at time 0, 24 h and 48 h and extracted for HPLC analyses of microalgal pigments for measurement of assemblage change with time after impact by the demolition charge. These subsamples are currently being analyzed. In addition, primary production was also measured in 24 and 48 h ambient light and temperature incubations. Primary production was stimulated after 24 h but returned to background levels within 48 h suggested a minimal long term impact of chemicals released in the detonation on the microalgal assemblage. Although impact of the TNT detonation on primary production appeared to be limited, there was stimulation of primary production in the samples post RDX detonation.

Task 4. Bacterial Degradation

Subtask 4.1 Determine metabolic efficiency of NEC degradation

During the estuarine sediment surveys, we found that TNT mineralization was frequently more rapid than 2,4-DNT or 2,6-DAT mineralization at the same station and time point. This could be due to a greater percentage of the bacterial community metabolizing TNT, more inefficient use of TNT by bacteria (lower % incorporation), or different cellular transport mechanism for TNT versus 2,4-DNT resulting in greater uptake and incorporation of TNT.

Estuarine surface water (salinity = 30 ‰) was collected from the mouth of the Delaware Bay in March 2005 and incubated with ^{14}C-TNT, ^{14}C-DNT and ^{14}C-DAT to measure the incorporation and mineralization rates and growth efficiencies of free-living bacteria metabolizing these compounds. Bacteria among the natural assemblage incorporated TNT and 2,6-DAT into macromolecules (TCA precipitate) at a similar rate over the course of the 24 h incubation (28.8 +/- 12.2 versus 24.5 +/- 2.2 μg C L^{-1} d^{-1}, respectively) but had a lower growth efficiency on TNT than 2,6-DAT and the mineralization rate was approximately six fold less for 2,6-DAT than TNT (3.1 +/- 0.9 versus 0.5 +/- 0.2 μg C L^{-1} d^{-1})(Table 1). That is, heterotrophic bacteria processed 2,6-DAT and TNT at about the same rate, but more of the 2,6-DAT carbon was incorporated into macromolecules and more the TNT carbon was respired as CO_2.

At 3.2 (+/- 0.2) μg C L^{-1} d^{-1}, the incorporation rate of 2,4-DNT was almost an order of magnitude lower than that for both TNT and 2,6-DAT (Table 1). However, at 1.1 (+/- 0.7) μg C L^{-1} d^{-1}, 2,4-DNT mineralization rate was only about three-fold lower than that for TNT and about twice that for 2,6-DAT due to a much lower growth efficiency for bacterial metabolism of 2,4-DNT (74.4%). Bacteria incorporated a much lower percentage of the 2,4-DNT carbon than they did for 2,6-DAT and TNT. Though there were differences in incorporation efficiencies among these compounds, they are still surprisingly high for organic carbon sources metabolized by a natural assemblage. One hypothesis generated from these results is that these compounds are metabolized efficiently because the N-C subunits of NEC are easily transformed into macromolecules like proteins and nucleic acids. Assimilatory nitrogen metabolism by bacteria may be the preferred transformation process over than organic carbon oxidation of the aromatic ring for energy. Although the mineralization rates (μg C L^{-1} d^{-1}) were high relative to other organic carbon substrates, the percentage of assimilated TNT carbon that was mineralized was so low (ca. 10%) that it may actually be due to remineralization of bacterially incorporated ^{14}C by protozoan grazers. We will investigate this possibility during the next calendar year.

Table 1. Rates of TNT incorporation, mineralization (μg C kg^{-1} d^{-1}) and bacterial growth efficiency (%) using Delaware Bay surface water collected March 2005.

Energetic	Rate (μg C L^{-1} d^{-1})		% Growth Efficiency
	Incorporation	Mineralization	
TNT	28.8 (12.2)	3.1 (0.9)	90.3
DAT	24.5 (2.2)	0.5 (0.2)	98.0
DNT	3.2 (0.2)	1.1 (0.7)	74.4

Subtask 4.2 Field surveys of NEC degradation rates in water and sediment

Currently, the progress in this subtask involves two areas: NEC mineralization rates (^{14}C-TNT, -DNT, -DAT) determined for estuarine and marine surface sediments; and, RDX, HMX and 2,4-DNT degradation rates using cultured marine bacteria isolated from an unexploded ordnance site offshore of Oahu. In addition to the data collected as part of the Chesapeake-Delaware Bay and Hawaii samplings, *Walker et al.* (2006) compares data from seven other

estuarine and marine sampling events (ONR and SERDP funded). For these comparisons, the detection limit of our 24 h ^{14}C-radiotracer incubations with natural sediment samples (1 g wet volume) was 1.0 x 10^{-2} μg C kg^{-1} d^{-1} though a sample was only considered to have detectable NEC mineralization rates if the average of the triplicate live values (minus the kill) was greater than the standard deviation. If the standard deviation was larger than the average mineralization value of the triplicate samples, then that station was listed as a nondetect for TNT mineralization.

In general, TNT mineralization rates by the natural bacterial assemblage in surface sediments were higher than 2,4-DNT and 2,6-DAT mineralization rates measured at the same station. TNT mineralization was detected at 40 out of 56 stations (71%) and ranged from nondetect to 145 (+/- 16.0) μg C kg^{-1} d^{-1} though the median values of the each survey only ranged from 3.0 (+/- 0.56) to 17.6 (+/- 0.49) μg C kg^{-1} d^{-1} (Table 2). These median values may be a more useful for determining the capacity of ecosystem sediments to metabolize TNT. Despite the wide variation in ecosystem types (tropical versus temperate), the median TNT mineralization rates were within an order of magnitude among surveys. In addition, mineralization of 2,4-DNT and 2,6-DAT were also measured in the Chesapeake Bay surveys and the assays were typically conducted at the same stations as those for TNT mineralization. 2,4-DNT and 2,6-DAT mineralization was detected at fewer stations (38% and 58%, respectively) and had a lower median range for stations where mineralization was detected (1.35 +/- 0.44 and 9.88 +/- 0.48 μg C kg^{-1} d^{-1}, respectively). In San Francisco Bay, only one survey was performed (May 2003) and TNT mineralization was detected at four out of 10 stations with 2,4-DNT mineralization detected at two out of 10 stations. The Hawaii sampling involved surveys of Bishop's Point and South Loch in Pearl Harbor and Ala Wai Canal in Waikiki with 45% of the 11 samples having detectable TNT mineralization rates (median rate = 3.57 +/- 1.51 μg C kg^{-1} d^{-1})(*Montgomery and Osburn* 2004). When averaged across all data for a sampling cruise, TNT mineralization rates in the Chesapeake Bay were higher than those measured for Hawaii and San Francisco Bay.

Table 2. Nitrogenous energetic compound (NEC) mineralization rates (μg C kg^{-1} d^{-1}) were surveyed for surface sediments in three coastal waterways from 2002 to 2005. The range and median rate are reported for the collection of samples in each ecosystem where mineralization was detected.

Ecosystem (# sampling events)	NEC	Total Stations	Detects	Mineralization Range AVG μg C kg^{-1} d^{-1} (+/-SD)		Median Rate for Detects AVG (+/-SD)
				Low	High	
Chesapeake Bay (3)	TNT	35	31	0.29 (+/-0.13)	145 (+/-16.0)	17.6 (+/-0.49)
	DNT	26	10	0.14 (+/-0.05)	219 (+-119)	1.35 (+/-0.44)
	DAT	17	10	2.15 (+/-0.30)	49.3 (+/-22.6)	9.88 (+/-0.48)
San Francisco Bay (1)	TNT	10	4	2.96 (+/-1.33)	7.42 (+/-2.11)	3.08 (+/-0.56)
	DNT	12	2	16.0 (+/-12.4)	68.2 (+/-12.8)	Not Applicable
Hawaii (2)	TNT	11	5	2.38 (+/-1.60)	19.9 (+/-11.8)	3.47 (+/-1.51)

The bacterial assemblage degradation of NEC may be to metabolism of other aromatic compounds like PAHs (^{14}C-naphthalene, -phenanthrene, -fluoranthene), toluene, catechol, or even heterotrophic metabolism in general (bacterial production). Given the wide range of

ecosystems and different times of the year of our samplings, we did not find good correlations when comparing TNT mineralization rates with these other parameters on a station by station basis. However, we did find that when averaging rates between stations where TNT mineralization was detected versus not detected, the average rates of bacterial production and mineralization of naphthalene, phenanthrene, fluoranthene, catechol and toluene were all higher at stations where TNT mineralization was detected (Table 3). Also, the range in average PAH and toluene mineralization rates for all the stations (0.80 to 8.04 μg C kg^{-1} d^{-1}) is very similar to that for TNT (3.08 to 17.6 μg C kg^{-1} d^{-1}). This suggests that TNT is mineralized in coastal sediments at rates that are typical for naturally occurring compounds, like phenanthrene and fluoranthene, and even similar to compounds like toluene and naphthalene that are known to be very transient in the estuarine sediments. 2,6-DAT mineralization rates (AVG) were higher stations where TNT mineralization was detected (9.00 versus 3.84 μg C kg^{-1} d^{-1}), but 2,4-DNT mineralization rates (AVG) were actually lower (13.8 versus 10.3 μg C kg^{-1} d^{-1}). This suggesting that TNT and 2,4-DNT mineralization may be uncoupled processes amongst the natural assemblage and that is possible that different components of the assemblage metabolize these two different compounds. Alternatively, their metabolism by bacteria is affected in different ways by environmental conditions.

Table 3. TNT mineralization was measured on sediment samples (68 total) from the Chesapeake Bay (35), San Francisco Bay (10) and the Ala Wai canal, HI (11). Stations with detectable TNT mineralization rates were compared with those that were below the detection limit. Bacterial production and mineralization rates (μg C kg^{-1} d^{-1}) for other organic compounds were generally higher for samples that had measurable TNT mineralization with the notable exception of 2,4-DNT.

TNT Mineralization Rate	Production or Mineralization Rate (μg C kg^{-1} d^{-1})							
	Bacterial production	Naphthalene	Phenanthrene	Fluoranthene	Catechol	DNT	DAT	Toluene
Detects (42)	76.3	4.79	8.04	1.06	160	10.3	9.00	4.45
Non-detects (26)	10.1	1.40	4.66	0.80	113	13.8	3.84	3.81

In addition to the field work on natural assemblages, laboratory experiments were performed using bacterial monocultures isolated from impacted marine environments. One marine bacterium, *Clostridium* sp. EDB2, was isolated from a UXO field offshore of Oahu and could degrade cyclic nitramines in the presence of Fe(III), humic acids (HA), and anthraquinone-2,6-disulphonate (AQDS). This obligate anaerobe degraded RDX and HMX to nitrite, methylenedinitramine, nitrous oxide, ammonium, formaldehyde, formic acid, and carbon dioxide. Carbon and nitrogen mass-balance for RDX products were 87 and 82%, respectively, and 88 and 74% for HMX products, respectively, suggesting that incorporation efficiencies ranged from 12 to 26%. Bacterial growth and biodegradation of RDX and HMX were stimulated in the presence of Fe(III), HA, and AQDS suggesting that strain EDB2 utilized Fe(III), HA, and AQDS as redox-mediators to transfer electrons to cyclic nitramines. This strain demonstrated a multidimensional approach to degrading RDX and HMX: first, direct degradation of the parent compound; second, indirect degradation by reducing Fe(III) to produce reactive-Fe(II); and third, indirect degradation by reducing HA and AQDS which act as electron-shuttles to transfer electrons to the cyclic-nitramines. A portion of this work was submitted for publication in a peer-reviewed journal (*Bhushan et al.* 2005).

Laboratory work was also performed to understand the bacterial biodegradation of 2,4-DNT by a novel marine bacterium, *Pseudoxanthomonas* sp. JA40, isolated from the Johnston Atoll in the Pacific Ocean. JA40 is hypothesized to degrade 2,4-DNT because it evolved in an environment deficient of organic nitrogen sources but rich in PAHs and polychlorinated biphenyls (PCBs). This facultative anaerobe was able to transform 2,4-DNT both aerobically (with the production of metabolites) and anaerobically, using this NEC as both a carbon and nitrogen source. This work was submitted for publication in a peer-reviewed journal (*Cheng et al.* 2006).

RDX and HMX were degraded using anaerobic enrichment cultures from the marine sediment samples from the Oahu UXO field. These mixed cultures mineralized 69% or 57% (25 days) of the total carbon of ^{14}C-RDX or ^{14}C-HMX, respectively. The most abundant members of the consortium (based on PCR-DGGE) were γ-proteobacteria (*Halomonas*), sulfate-reducing δ-proteobacteria (*Desulfovibrio*), firmicutes (*Clostridium*), and fusobacterium. Among 22 sediment bacterial isolates screened for RDX and HMX biodegradation activity under anaerobic conditions, 5 were positive for RDX and identified as *Halomonas, Marinobacter, Pseudoalteromonas* and *Bacillus* based on 16S rRNA. Sedimentary bacteria degraded RDX to N_2O and HCHO via the intermediary formation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and methylenedinitramine. This work was submitted for publication in a peer-reviewed journal (*Bhatt et al.* 2005).

Subtask 4.3 Sample seawater during and after subsurface detonation

Even though NECs were not detected in the plume samples, we were still able to measure some effects of the detonations on the natural microbial assemblage. Bacterial production appeared to be unchanged following the TNT detonation when coming surface seawater taken pre verse post-detonation whereas there was an increase in bacterial production in the plume related to the RDX detonation (Table 4). Even though we were unable to detect NEC present in the plume seawater samples, it is possible that decomposition of the RDX resulting from the detonation could have resulted in a nutrient release (i.e. nitrate) which could stimulate heterotrophic bacterial metabolism. TNT uptake and mineralization was measured using radiotracer addition to the subsamples of the incubations. Light appeared to increase uptake of TNT in roughly the same relative amount that light increased bacterial production (Table 5). There was no obvious change in bacterial assemblage composition over the 48 h of the incubations, as determined by DGGE profiles of extracted DNA. Our current work and previous ecosystem surveys of TNT and 2,4-DNT mineralization in surface sediments and with depth in sediment cores was invited for a platform presentation at SETAC in Baltimore, MD (*Montgomery et al.* 2005) and is found in the Formal Report (*Walker et al.* 2005).

Table 4. Bacterial production in seawater samples collected pre- and post-detonation of TNT and RDX. There was little difference in bacterial production (μg C L^{-1} d^{-1}) as a result of the detonation of TNT, but production roughly doubled in the post RDX detonation sample incubated in either ambient light or dark.

Energetic	Illumination	Detonation	Bacterial Production (μg C L^{-1} d^{-1})	
			AVG	S.D.
TNT	Light	Pre	3.36	0.03
		Post	3.33	0.37
	Dark	Pre	2.06	0.28
		Post	1.73	0.63
RDX	Light	Pre	1.83	0.30
		Post	4.77	1.42
	Dark	Pre	1.27	0.79
		Post	2.32	0.19

Table 5. TNT uptake and mineralization rates by bacteria in seawater samples collected pre- and post-detonation of TNT. There was little difference in bacterial production, TNT mineralization or uptake rate (μg C L^{-1} d^{-1}) as a result of the detonation. Incubation under ambient light increased both bacterial production and TNT uptake (μg C L^{-1} d^{-1}); mineralization was not measured under dark conditions.

Illumination	Detonation	Bacterial Production (μg C L^{-1} d^{-1})		Rate of TNT Uptake (μg C L^{-1} d^{-1})	Rate of TNT Mineralization (μg C L^{-1} d^{-1})	
		AVG	S.D.	Range	AVG	SD
Light	Pre	3.36	0.03	161-170	5.00	2.55
	Post	3.33	0.37	231-241	4.99	2.39
Dark	Pre	2.06	0.28	71-123		
	Post	1.73	0.63	88-100		

Conclusions

Thus far, it appears as though abiotic hydrolysis of NEC in seawater occurs at a rate that is orders of magnitude slower (years) than transformation rates by culturable, anaerobic bacteria and than mineralization rates by natural microbial assemblages (days). The second year of this project will focus more on the mechanisms of microbial degradation of NEC as well as the comparison to the rates of photodegradation. Preliminarily, it appears that natural attenuation processes have the potential to remove NEC released into the marine environment on a time scale that may be useful in standard risk assessment analyses of impacted sites.

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